1560

GELATIN DEGRADATION BY MICROBIAL AND OTHER ENZYMES AS RELATED TO THEIR UNHAIRING ACTIVITY*

H. W. JONES, T. C. CORDON, J. NAGHSKI, AND W. WINDUS†

Eastern Regional Research Laboratory‡
Philadelphia 18, Pennsylvania

ABSTRACT

A study of the enzymatic depilation of cattlehide would be aided materially if an assay method could be found which correlated with hair-loosening action. Enzymes from bacteria, fungi, plants, and animals were evaluated according to their action on gelatin as measured by formol titration, decrease in viscosity, and liquefaction. Although the correlation between these assay methods and the degree of hair loosening was statistically significant, the magnitude of the relationship was not great enough to permit dependable predictions.

INTRODUCTION

In a study of the enzymatic depilation of hides and skins attempts have been made to find an assay procedure which would correlate with the depilatory action of the enzymes. Such an assay would greatly facilitate the work on unhairing and would lead to a better understanding of the mechanism of the depilatory action. Attempts to correlate hair-loosening activity of enzymes with action on casein and starch (1) and elastin (2) have been reported previously. Although neither gelatin nor its precursor, collagen, has been implicated in the depilatory action of enzymes, some workers believe that there may be a correlation between the ability of some enzymes to attack gelatin and their ability to cause the loosening of hair on animal hides. In order to check this we have assayed enzymes of plant, animal, and microbial origin by three different procedures using gelatin as the substrate. The results are reported here.

*Presented in part at the 60th Annual Meeting of the Society of American Bacteriologists, Philadelphia, Pennsylvania, May 1–5, 1960.
†Life Lines of all the authors were published in last month's *Journal*.
‡Eastern Utilization Research and Development Division, Agricultural Research Service, U. S. Department of Agriculture

MATERIALS

Enzymes.—The enzymes studied were supplied by the firms listed in Table I. The abbreviations given there are also used in the tables that follow. These enzymes were used as received without further purification.

TABLE I

LIST OF THE SUPPLIERS OF THE ENZYMES USED IN THESE STUDIES

Code	Source of Enzyme
MC	Miles Chemical Co., Clifton, N. J.
RH	Rohm & Haas Co., Philadelphia, Pa.
PB	Pabst Brewing Co., Milwaukee, Wis.
BS	Biddle-Sawyer Co., New York, N. Y.
NB	Nutritional Biochemicals Corp., Cleveland, Ohio
VC	Viobin Corporation, Monticello, Ill.
WC	Wallerstein Co., Staten Island, N. Y.
IM	Institute of Microbiology, New Brunswick, N. J.

Gelatin.—In order to obtain reproducible results it was necessary to use gelatin from one source. Even different lots from the same manufacturer had to be carefully checked. Gelatin supplied by the Amend Drug and Chemical Company* was used in all three assays.

Buffers.—The following buffers were used for maintaining the pH at the desired values:

pH 5.0 McIlvaine's citric acid–phosphate
pH 7.0 Mixture of $Na_2H\ PO_4$ and NaH_2PO_4
pH 8.5 Tris (hydroxymethyl) amino methane

The pH was held within about 0.1 unit throughout the reactions.

Formol titration.—In the formol titration assay 0.5-g. portions of gelatin were weighed into 50-ml. Erlenmeyer flasks, and 8 ml. of water and 2 ml. of buffer were added. The flasks were held in a water bath at 40°C. until the gelatin dissolved. Five ml. of enzyme solution was then added, and incubation was continued at 40°C. for one hour. Ten ml. of 37% neutralized formaldehyde was then added. In the case of the blanks the formaldehyde was added before the enzyme. The solutions were titrated to a faint pink color, using 0.1*N* NaOH and phenolphthalein as the indicator. Results are reported as milliequivalents of nitrogen per g. of enzyme. At pH values other than 7.0 the procedure was modified to the extent that gelatin solutions were adjusted to the desired pH with the buffer and aliquots pipetted into the flasks.

*The mention of trade names or companies does not constitute an endorsement by the Department of Agriculture over other products or companies not mentioned.

Liquefaction.—In this procedure gelatin solutions were made in the buffer solutions so that the concentration in the reaction mixture (33 mg/ml) was the same as that used in the formol titration assay. Enzyme solutions were diluted in 0.1% intervals, and one ml. from each dilution was placed in each of two test tubes. Four ml. of gelatin solution was added to each tube, and the tubes were held in a water bath at 40°C. for one hour. They were then placed in ice water, and after about ½ hour the concentration of enzyme in the last tube remaining liquid was recorded. In order to facilitate comparison with the other assay results the reciprocal of this concentration in micrograms per liter was recorded in the tables.

Viscometric assay.—A 4% gelatin solution was made up in water saturated with toluene. Ten ml. of this was placed in a stoppered flask in a 40° ± 0.5°C. water bath, and 4 ml. of buffer was added. When the solution reached 40°C., one ml. of enzyme was added, and the time was noted. Ten ml. of this mixture was immediately pipetted into the wide arm of an Ostwald-Fenske-Cannon-type viscosimeter, and measurements were begun after 2½ to 3 minutes of digestion time. Four successive determinations of efflux time were made in not more than a total of 15 minutes from the moment that the enzyme solution was added. Blanks were run in the same way except that water was added instead of the enzyme. The results were plotted, and relative fluidity values were obtained from the curve at 5- and 10-minute digestion times (Fr_5 and Fr_{10} respectively). The units of activity were obtained by using the formula

$$Units/g. = \frac{Fr_{10} - Fr_5 \times 33.3}{e}$$

where e = mg. enzyme contained in 1 ml. enzyme solution as added to the gelatin solution. This procedure is empirical, and all variables must be carefully controlled.

RESULTS

In order to simplify the interpretation of the data the ratios of the values for the three assays as well as the actual values obtained are given in Tables II, III, IV, and V. If each procedure measured the same activity of the enzymes, the ratios of the values obtained by the different assays should be constant. Conversely, divergence from constant values would indicate that the procedures were measuring different activities of the enzymes. The division of the enzymes into bacterial, fungal, and plant and animal groups was done merely to simplify presentation.

Table II shows the data obtained with five of the bacterial enzyme preparations. In considering the marked differences in the activities, it should be

TABLE II

COMPARISON OF THE ACTION OF SOME BACTERIAL ENZYMES ON GELATIN AT pH 7.0

Enzyme	Code	Assays			Ratios		
		Formol Titration meq-N/g	Viscosity Units/g	Liquefaction $\frac{1}{\mu g/l}$	V/FT	V/L	FT/L
Protease 15 Concentrate	RH	44.2	81.0	20.0	1.8	4.1	2.2
Protease L-56-D	PB	38.8	68.5	25.0	1.8	2.7	1.6
Enzyme 4511-3	WC	23.0	19.0	11.0	0.8	1.7	2.1
Rhozyme H-39	RH	16.5	6.2	8.6	0.4	0.7	1.9
Bioprase	BS	8.4	0.8	4.2	0.9	0.2	2.0

borne in mind that these relative activities are dependent upon the degree of purification of the enzyme preparation, and this varied with the intended end use for a particular product. Examination of the columns giving ratios of the assay values shows marked differences between the products. The values for the ratio of viscosity to formol titration vary as much as 4.5 times, and those for viscosity to liquefaction vary over 20 times. The ratios of formol titration to liquefaction are much more consistent. The greatest difference is 1.4 times between Protease 15 Concentrate and L-56-D.

TABLE III

COMPARISON OF THE ACTION OF BACTERIAL ENZYMES FROM THE SAME SOURCE (CODE MC) ON GELATIN AT pH 7.0

Enzyme	Assays			Ratios		
	Formol Titration meq-N/g	Viscosity Units/g	Liquefaction $\frac{1}{\mu g/l}$	V/FT	V/L	FT/L
HT Proteolytic, 202	96.9	197.5	62.5	2.0	3.2	1.6
HT Concentrate "P"	94.9	165.5	33.3	1.7	5.0	2.9
HT Concentrate, 4903	71.3	123.0	50.0	1.7	2.5	1.4
HT Proteolytic, 1	55.2	136.5	33.3	2.5	4.1	1.7
HT Proteolytic, 210	53.4	88.0	20.0	1.7	4.4	2.7
HT Proteolytic, 207	41.2	90.0	20.0	2.2	4.5	2.1

Table III gives the data obtained with six bacterial enzyme preparations all from the same organism but not necessarily produced under the same cultural conditions. As a group these preparations are much more potent than

those listed in the previous table. According to the assay in Northrup Units supplied by the manufacturer the first two preparations listed are about double the strength of the other four, about 930 to about 420 NU/g. respectively. None of our assays agrees very well with these values. The ratios of the values obtained by our assays are in fair agreement with those for the other bacterial enzymes, but even here there are over twofold differences.

Data for the new fungal enzymes tested are given in Table IV. Keratinase which is produced by *Streptomyces fradiae* is included for convenience. Considerable activity is shown by these preparations, but they are not as potent

TABLE IV

COMPARISON OF THE ACTION OF SOME FUNGAL ENZYMES ON GELATIN AT pH 7.0

Enzyme	Code	Assays			Ratios		
		Formol Titration meq-N/g	Viscosity Units/g	Liquefaction $\frac{1}{\mu g/l}$	V/FT	V/L	FT/L
Fungal Protease	PB	79.9	29.0	33.3	0.4	0.9	2.4
Rhozyme PII	RH	34.3	14.5	16.6	0.4	0.9	2.1
Prolase 40	WC	31.8	12.5	10.0	0.4	1.3	3 2
Keratinase	IM	70.7	32.0	50.0	0.5	0.6	1.4
Keratinase (at pH 8.5)	IM	125.1	51.0	50.0	0.4	1.0	2.5

as several of the bacterial enzymes. The ratios of the various activities are fairly consistent, but some differences are apparent. The optimum pH for the keratolytic activity of Keratinase is 8.5 to 9.0, and it is also more active on gelatin at pH 8.5 than 7.0 except when measured by the liquefaction technique.

Table V gives the data obtained using enzymes of plant and animal origin. Bromelin has comparatively high activity, but papain is quite weak. Special Diastase 160 is a mixture of plant and animal enzymes, mostly amylolytic, and has only weak action on gelatin. Crystalline trypsin digests gelatin more actively than any of the other enzyme preparations tested. However, the increase of activity over the noncrystalline pancreatic preparations varied markedly depending upon the assay method. Again there was much discrepancy between the ratios of the values for the three assays. It is well known that the optimum pH for the action of pancreatic enzymes is above 8.0. Therefore, in addition to those reported in this Table, assays were run on the three tryptic preparations at pH 8.5. The increased activities at pH 8.5 over their activities at pH 7.0 varied from 8 to 30%.

TABLE V

COMPARISON OF THE ACTION OF PLANT AND ANIMAL ENZYMES ON GELATIN AT pH 7.0

Enzyme	Code	Assays			Ratios		
		Formol Titration meq-N/g	Viscosity Units/g	Liquefaction $\frac{1}{\mu g/l}$	V/FT	V/L	FT/L
Bromelin	MC	77.7	67.0	100.0	0.9	0.7	0.8
Papain	NB	14.2	7.7	6.3	0.5	1.2	2.3
Special Diastase 160	MC	6.7	0.7	1.3	0.1	0.6	5.4
Viokase	VC	46.0	3.5	16.0	0.1	0.2	2 9
Trypsin (4 × USP Pancreatin)	NB	50.8	2.5	25.0	0.1	0.1	2.0
Trypsin (crystalline)	NB	435.2	750.0	500.0	1.7	1.5	0.9

TABLE VI

THE EFFECT OF pH AND ACTIVATOR ON THE ACTION OF BROMELIN AND PAPAIN ON GELATIN

pH	Sulfite* %	Bromelin (MC)		Papain (NB)	
		Formol Titration meq-N/g	Liquefaction $\frac{1}{\mu g/l}$	Formol Titration meg-N/g	Liquefaction $\frac{1}{\mu g/l}$
5.0	None	178.8	66.6	22.4	0.6
5.0	0.4	46.1	33.3	4.0	0.1
7.0	None	77.7	100.0	14.2	6.3
7.0	0.4	81.6	83.3	4.6	20.8
8.5	None	—	28.6	—	5.0
8.5	0.4	—	83.3	—	16.6

*As sodium metabisulfite on total solution basis

The effects of controlling the pH at 5.0, 7.0, and 8.5 and the presence of sodium metabisulfite on the activity of bromelin and papain are shown in Table VI. According to the formol titration assay bromelin was 2.3 times more active at pH 5.0 than at pH 7.0. Sulfite exhibited a marked inhibitory action at pH 5.0 and a slight activation at pH 7.0. We were unable to obtain reliable values at pH 8.5. Results using the liquefaction assay show that the highest activity for bromelin without activator was at pH 7.0. The addition

of sulfite resulted in a 50% decrease of activity at pH 5.0, a slight decrease at pH 7.0, and a threefold increase at 8.5.

Papain was much less active than bromelin. According to the formol titration assay the highest activity of papain was at pH 5.0. Sulfite had a decided inhibitory effect at both pH 5.0 and 7.0. Results with the liquefaction assay showed that there was very little activity at pH 5.0 and only moderate activity at pH 7.0 and pH 8.5. With this assay the presence of sulfite caused an inhibition at pH 5.0 but marked activation at pH 7.0 and pH 8.5.

The comparative depilatory activities of these enzymes have been determined and reported previously (1, 2, 3). The unhairing scores of the enzymes are compared in Table VII with their relative ranks according to the three

TABLE VII

COMPARISON OF THE UNHAIRING SCORES* OF ENZYMES WITH THEIR RELATIVE RANKS ACCORDING TO ASSAYS BY FORMOL TITRATION, VISCOSITY, AND LIQUEFACTION

Enzyme	Code	Unhairing Score	Formol Titration	Viscosity	Liquefaction
Trypsin (Crystalline)	NB	—	1	1	1
HT Proteolytic, 202	MC	1	3	2	3
HT Proteolytic "P"	MC	1	4	3	5
Keratinase (pH 8.5)	IM	1†	2	11	4
HT Concentrate, 4903	MC	2	7	5	4
HT Proteolytic, 1	MC	2	8	4	5
HT Proteolytic, 207	MC	2	13	6	7
HT Proteolytic, 210	MC	3	9	7	7
Protease L56-D	PB	3	14	9	6
Keratinase (pH 7.0)	IM	4†	7	12	4
Protease 15 Concentrate	RH	5	12	8	7
Trypsin (4 × USP Pancreatin)	NB	6	10	20	6
Protease 4511-3	WC	7	17	14	10
Fungal Protease	PB	8	5	13	5
Bromelin	MC	8	6	10	2
Rhozyme P11	RH	9	15	15	8
Prolase 40	WC	10	16	16	11
Papain	NB	11	19	17	13
Viokase	VC	11	11	19	9
Special Diastase 160	MC	12	21	21	15
Rhozyme H39	RH	13	18	18	12

*From Cordon *et al.* (2)

†Estimated from many tests. This enzyme was used on the basis of a keratinase assay (5) and cannot be compared on a weight basis with the other enzymes.

gelatin assays. Crystalline trypsin far outranks all other preparations in the gelatinase assays. However, because of its high cost, its use as an unhairing agent is precluded, and even the testing of its hair-loosening ability was considered impractical. When the methods of assay were tested in comparison with the unhairing activity, they were significant statistically. However, the magnitude of the relationship was not great enough to permit dependable predictions. In the best case tested (bacterial enzymes against viscosity) it was only possible to estimate the unhairing activity within ± 40% with 95% confidence.

DISCUSSION

Although enzymatic attack on gelatin undoubtedly consists of cleavage of the molecule, this is apparently accomplished in different ways by different enzymes. If breakdown always consisted of the rupture of the same linkages, the values obtained by titrating the acid produced and the change in viscosity or the ability to form a gel should be constant. Obviously this is not so. Perhaps changes somewhat analogous to those taking place when collagen is converted into gelatin by heating in water are produced by some of the enzyme systems.

At first thought it might appear that the viscosity assay and the liquefaction assay are measures of the same property of the gelatin. Liquefaction, however, as we define it, is really the ability of the protein to form a gel, and this is dependent on its ability to form a lattice network, a property quite different from viscosity. The data presented are replete with examples of enzyme preparations exhibiting the three different activities at different levels. Furthermore, alteration of the reaction conditions may or may not affect the three types of activity to the same extent when the pH is raised. A case in point is the change observed with Keratinase when the pH is raised from 7.0 to 8.5. This causes a 1.8-fold increase in activity as measured by formol titration, a 1.6-fold increase in activity as measured by viscosity, but no change in activity as measured by liquefaction.

Further evidence that different activities of the enzyme preparations are measured by the different assays is shown by the effects of changing the pH and adding a reducing agent to bromelin. pH 5.0 appears to be at or near the optimum for the action as measured by formol titration, whereas 7.0 is near the optimum as measured by liquefaction. Why sulfite should cause such a pronounced inhibition at pH 5.0 is not readily apparent unless the scission of the disulfide bond in bromelin leads to its complete conversion to the S-sulfo compound (4). Here again the effect is twice as pronounced with the formol titration as with the liquefaction assay.

The lack of correlation between hair loosening and any of the gelatinase assays clearly rules out this type of procedure for general use in studies of

the enzyme unhairing of hides and skins. However, if a single enzyme preparation were under investigation, one of the methods might prove useful for following its activity in unhairing studies.

SUMMARY

Twenty-one enzyme preparations were tested by three different assay procedures for their ability to attack gelatin. Results with the different procedures were quite variable. It was concluded that the different assay procedures measure different activities of the enzymes. No correlation was found between hair loosening and any of the assays.

ACKNOWLEDGMENT

The authors wish to thank Mr. C. E. Land of the Miles Chemical Company for supplying the viscosity assay method used and several of the enzyme suppliers for donating their products.

REFERENCES

1. Cordon, T. C., Jones, H. W., Clarke, I. D., and Naghski, J. *Appl. Microbial.*, **6**, 293 (1958).
2. ———, Everett, A. L., Jones, H. W., Windus, W., and Naghski, J. *JALCA*, **56**, 68 (1961).
3. ———, Windus, W., Clarke, I. D., and Naghski, J. *JALCA*, **54**, 122 (1959).
4. Swan, J. M. *Nature*, **180**, 643 (1957).

Received October 19, 1960.